29

PATENT SPECIFICATION

(11) 1243784

1243784

DRAWINGS ATTACHED

(21) Application No. 45046/67 (22) Filed 3 Oct. 1967
(21) Application No. 35921/68 (22) Filed 26 July 1968
(23) Complete Specification filed 20 Sept. 1968
(45) Complete Specification published 25 Aug. 1971
(51) International Classification C 12 d 13/10; C 02 c 1/00; C 11 d 1/02, 1/66, 1/84, 3/04, 3/38, 7/42; C 12 k 1/10
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C6F 1X
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C3H 3
C5D 6A5A 6A5B 6A5C 6A5D1 6A5D2 6A5E 6A8B 6B12A 6B12B1 6B12E 6B12G2A 6B12N2 6B8 6C6
C6C 2J3 2J4

(72) The inventors of this invention in the sense of being the actual devisers thereof within the meaning of Section 16 of The Patents Act 1949 are KNUD AUNSTRUP, OTTO ANDERSEN and HELLE OUTTRUP, all Danish subjects, of 82 Skovbakken, Farum, 67, Studie-straede, Copenhagen and 57 Bavne-stedet, Værlose, Denmark respectively.

(54) PROTEOLYTIC ENZYMES, THEIR PRODUCTION AND USE

(71) We, NOVO TERAPEUTISK LABORATORIUM A/S, a Danish Company of 115, Fuglebakkevej, Copenhagen, Denmark, do hereby declare the invention for which we pray that a patent may be granted to us, and

ERRATUM

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Page 13, Table IV, Item VI, 5th Column *for* 5.3 *read* 7.3

THE PATENT OFFICE
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temperature and/or stability in the presence of non-enzymatic substances being a constituent part of the enzyme-containing preparations or compositions.

It is known that proteolytic enzymes can be produced by cultivation of certain bacteria under aerobic conditions, but the proteolytic enzymes produced by the known cultivations show optimal proteolytic activity against hemoglobin at a pH-value which in the most favourable cases did not reach more than 9.

The present invention is based on the observation that there exists in nature a great number of hitherto unknown bacteria forming during their metabolism proteolytic enzymes which display optimal proteolytic activity against hemoglobin at high pH-

of from 50 to 80% and, in a third embodiment in the range of from 0 to 50%.

According to a second aspect of the present invention, there is provided a process for producing the proteolytic enzyme(s) contained in the preparation of the preceding paragraph by aerobic cultivation of bacteria in a nutrient medium containing assimilable carbon and nitrogen sources, which comprises the cultivation of protease-producing species of the genus Bacillus isolated from nature on nutrient media with a pH-value within the range of 9 to 11, the maintenance of the pH-

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(54) PROTEOLYTIC ENZYMES, THEIR PRODUCTION AND USE

(71) We, NOVO TERAPEUTISK LABORATORIUM A/S, a Danish Company of 115, Fuglebakkevej, Copenhagen, Denmark, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to preparations and compositions comprising new enzymes showing a pronounced and useful proteolytic activity at high alkalinities and to a process of preparing such enzymes.

Within various fields there is demand for proteolytic enzymes and in some of these fields it is important that the enzymes display optimal proteolytic activities at high pH-values up to 10 to 12 and even higher, and that the enzymes are in possession of other useful properties, such as stability at elevated temperature and/or stability in the presence of non-enzymatic substances being a constituent part of the enzyme-containing preparations or compositions.

It is known that proteolytic enzymes can be produced by cultivation of certain bacteria under aerobic conditions, but the proteolytic enzymes produced by the known cultivations show optimal proteolytic activity against hemoglobin at a pH-value which in the most favourable cases did not reach more than 9.

The present invention is based on the observation that there exists in nature a great number of hitherto unknown bacteria forming during their metabolism proteolytic enzymes which display optimal proteolytic activity against hemoglobin at high pH-values up to 10 to 12, and which have other properties making them excellently suited for use within different industrial fields.

According to one aspect of the present invention there is provided an enzyme preparation containing at east one proteolytic enzyme of the serine type produced by cultivation of species of the genus Bacillus, the said enzyme showing optimal proteolytic activity against hemoglobin in the presence of urea at a pH-value above 9. When the proteolytic activity of the enzyme is measured at pH 12 by the Anson-method, the value of proteolytic activity is, in one embodiment of the invention, in the range of from 80 to 100% of the maximum activity, in a second embodiment, in the range of from 50 to 80% and, in a third embodiment in the range of from 0 to 50%.

According to a second aspect of the present invention, there is provided a process for producing the proteolytic enzyme(s) contained in the preparation of the preceding paragraph by aerobic cultivation of bacteria in a nutrient medium containing assimilable carbon and nitrogen sources, which comprises the cultivation of protease-producing species of the genus Bacillus isolated from nature on nutrient media with a pH-value within the range of 9 to 11, the maintenance of the pH-

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value of the nutrient medium within the range of from 7 to 12 during the main period of the cultivation, and the recovery of the proteolytic enzyme(s) formed during the cultivation. The invention also provides enzymes produced by the said process.

From samples of soil, animal manure and a number of other sources in nature the inventors have isolated about one hundred strains of bacteria, carried out taxonomic investigations and found that all of the hitherto unknown bacteria belong to the genus Bacillus, but that none of them belonged to any species known to the inventors, and that, to the best of the inventors' knowledge, they did not belong to the same species. Furthermore, within the same species there were in most cases different strains and several varieties.

For the purpose of isolating the hitherto unknown bacteria referred to above, use has been made of a novel technique which is characterized by the fact that the isolation is effected on nutrient media having a pH-value within the range of 9 to 11, and intended for detection of production of proteolytic enzymes.

In other words, the samples of soil, animal manure or other sources from nature have been spread on nutrient media having the high pH-value referred to and the bacteria able to grow under such alkaline conditions are then isolated and subjected to further investigations as to species and enzyme production.

In most cases, use has, according to the invention, also been made of a number of different enrichment methods.

Enrichment methods are known in the art. Reference can be made to

Hayaishi, Methods in Enzymology, Vol. 1, 126—131. One principle is to let a sample from nature grow on a nutrient medium having a specific and selected composition favouring the growth of a microorganism giving metabolic products having the properties aimed at. Another principle is to store the sample from nature together with a compound, such as an inorganic salt, favouring the development of the desired microorganism, cf. M.A. El-Nakeeb and H.A. Lechevalier, Appl. Microbiol., Vol. 11, 75 (1963), and thereafter to spread the sample on a suitable nutrient medium adjusted to a pH-value within the range of from 9 to 11.

Some of the hitherto unknown members of the genus Bacillus which have been isolated and tested taxonomically and for production of proteolytic enzymes are compiled in Table I below, in which the first column contains the inventors' reference number, the second column the number under which the bacterium has been deposited at The National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, the third colmn the source of isolation, and the fourth colmn the enrichment method used.

TABLE 1

Ref. No.:	Number NCIB:	Source of Isolation:	Enrichment Method:
C 300	10144	Soil from cemetery in Copenhagen	Starch-casein medium (pH raised stepwise from 10 to 12)
C 301	10145	Soil from cemetery in Copenhagen	Spreading of soil samples on agar with sesquicarbonate (pH = 9.6 — 9.8). Testing of zones of hydrolysis on neutral agar with skim milk
C 302	10146	Soil from Copenhagener river bank	
C 303	10147	Heap of soil and leaves from cemetry in Copenhagen	
C 304	10148	Wood sand from Blokhus Jutland	
C 311	10281	Wood soil from Ascheberg, Holstein	Perborate agar
C 323	10282	Field soil from Danish town	Perborate agar
C 324	10283	Lake bank soil from Ascheberg, Holstein	Perborate agar

TABLE I (Continued)

Ref. No.	Number NCIB:	Source of Isolation:	Enrichment Method:
C 325	10284	Infection on plate with perborate holes	Alkaline skim milk agar plates with holes filled with sodium perborate
C 326	10285	Infection on plate with perborate holes	
C 334	10286	Bank river soil from Danish town	Perborate agar
C 335	10287	Garden soil from Danish town	Perborate agar
C 336	10288	Horse and elephant manure	Perborate agar
C 337	10289	Clay from grass field from Ascheberg, Holstein	Perborate storing
C 338	10290	Soil from cemetery in Copenhagen	Perborate storing
C 339	10291	Bank river soil from Danish town	Perborate agar
C 340	10292	Bank river soil from Danish town	Perborate agar
C 341	10293	Field soil from Danish town	Perborate agar
C 342	10294	Garden soil from Danish village	Sodium Carbonate storing
C 343	10295	Garden soil from Danish town	Perborate agar
C 346	10296	Chicken yard soil from Danish town	Multiple alkaline starch enrichment
C 347	10297	Deer manure from deer park near Copenhagen	Multiple alkaline starch enrichment
C 348	10298	Chicken run soil	Multiple alkaline starch enrichment
C 349	10299	Deer manure from deer park near Copenhagen	
C 350	10300	Water from Copenhagener lake	Alkaline starch-casein medium with tripolyphosphate
C 351	10301	Chicken manure	Thermophile sesquicarbonate enrichment (50 °C., pH 8.8 to 9.7)

TABLE I (Continued)

Ref. No.	Number NCIB:	Source of Isolation:	Enrichment Method:
C 352	10302	Ostrich manure from Zoo	Sodium sesquicarbonate enrichment (pH 9.2—9.6)
C 353	10303	Manure from elephant	Sodium sesquicarbonate enrichment (pH: 9.2—9.6)
C 354	10304	Chicken yard soil	Enrichment on basic glucose nitrate at 40°C.
C 355	10305	Chicken yard soil	Enrichment on basic glucose nitrate at 40°C
C 356	10306	Garden bark	Enrichment on basic glucose nitrate at 50°C
C 357	10307	Chicken yard soil	Enrichment on basic glucose nitrate at 50°C
C 358	10308	Chicken yard soil	Enrichment on basic glucose nitrate at 50°C
C 360	10309	Garden soil from Danish town	Perborate agar
C 364	10310	Lavatory cistern scraping	Thermophile sesquicarbonate enrichment (50°C, pH: 8.8 — 9.7)
C 365	10311	Liquid from tannery liming bath	Bran-soda enrichment
C 366	10312	Baby faeces	Starch enrichment (pH 11) with inorganic nitrogen
C 367	10313	Manure from elephant	Thermophile sesquicarbonate enrichment (50°C, pH: 8.8 — 9.7)
C 369	10314	Ostrich manure from Zoo	Proteose peptone (shake flasks) pH 9.7
C 370	10315	Scraping from tannery liming bath containers	Alkaline mannitol—KNO_3 enrichment
C 371	10316	Manure from elephant	Proteose peptone (shake flasks) pH 9.7

TABLE 1 (Continued)

Ref. No.:	Number NCIB:	Source of Isolation:	Enrichment Method:
C 372	10317	Clay from grass field from Ascheberg, Holstein	Starch casein detergent enrichment
C 373	10318	Garden soil from Danish town	Perborate agar
C 374	10319	Clay from grass field from Ascheberg, Holstein	Ethylene diamine tetra the sodium salt of acetic acid-starch casein enrichment
C 375	10320	Ostrich manure from Zoo	Sodium sesquicarbonate enrichment (pH: 9.2—9.6)
C 376	10321	Manure from elephant	Sodium sesquicarbonate enrichment (pH: 9.2—9.6)
C 377	10322	Water from hippopotamus basin	Thermophile casein starch enrichment with NaOH
C 378	10323	Scraping from tannery liming bath containers	Mannitol-KNO_3 enrichment merit
C 410	10324	Tiger manure	Thermophile sesquicarbonate enrichment (50°C., pH: 8.8 — 9.7)
C 411	10325	Pigeon manure	
C 412	10326	Chicken yard soil from Danish town	Potato flour and sodium sesquicarbonate storing
C 413	10327	Clay from grass field from Ascheberg, Holstein	Starch enrichment (pH 11) with inorganic nitrogen

The taxonomic investigations of all these members of the genus Bacillus have been carried out while using the methods described by Smith, Gordon & Clark in "Aerobic Spore-forming Bacteria", U.S. Department of Agr., Monograph No. 16 (1952). These methods are up till now considered the most suitable ones, but they had to be modified in view of the fact that all nutrient media had to be adjusted on a much higher pH-value than that indicated by Smith, Gordon & Clark because all the Bacillus species listed in Table I grow at elevated pH-values.

The bacteria can be divided rather accurately into morphological groups. These groups differ from each other to such an extent that they actually represent separate species.

Within the morphological groups variations in the biochemical reactions are found. On the basis of these variations the groups have subdivided into varieties which are represented by one or more strains.

Species No. I
(belonging to morphological group I according to Bergey)

Morphology:
Vegetative rods: 0.5—0.7 $\mu \times 1.5$—4 μ
Spores: 0.5—0.8 $\mu \times 0.8$—1 μ
central to subterminal oval to cylindrical, thinwalled.
Sporangia: Very little, if any, swelling of spores.

Variety a:
C 300, C 301, C 360, C 372, C 374
Grampositive.
Growth on nutrient agar pH 7.3 as good as or better than on nutrient agar pH 9.7.
Maximum temperature for growth 50—55°C.
Scant growth, if any, on glucose- or mannitolagar pH 9.7 with nitrate as sole source of nitrogen.

Hydrolysis of starch: Positive, narrow zone of hydrolysis after 7 days.

Variety b:
C 302, C 334.
Grampositive.
Growth on nutrient agar pH 7.3 rather slow during the first two days, thereafter nearly comparable to growth on nutrient agar pH 9.7.
Maximum temperature for growth: 40—50°C.
Scant growth, if any, on glucose- or mannitolagar pH 9.7 with nitrate as sole source of nitrogen.
Hydrolysis of starch: Positive, wide zone of hydrolysis.

Variety c:
C 323, C 339, C 352, C 369.
Grampositive or Gramvariable.
Moderate to scant growth on nutrient agar pH 7.3.
Maximum temperature for growth 37—50°C.
Scant growth, if any, on glucose- or mannitolagar with nitrate as sole source of nitrogen.
Hydrolysis of starch: Positive, wide zone of hydrolysis.

Variety d:
C 304, C 311, C 336.
Grampositive rods.
Moderate to scant growth on nutrient agar at pH 7.3.
Maximum temperature for growth: 37°C.
Scant growth on glucose- or mannitolagar at pH 9.7 with nitrate as sole source of nitrogen.
Hydrolysis of starch: negative.

Species No. II
(belonging to morphological group I according to Bergey)
C 335, C 341.

Morphology:
Vegetative rods: 0.3—0.4 μ × 1.5—2.5 μ
Sports: 0.3—0.5 μ × 0.8—1 μ central to paracentral oval to cylindrical, thinwalled.
Sporangia: Very little, or no, swelling by spores.

Grampositive.
Almost no growth on nutrient agar at pH 7.3
Maximum temperature for growth: 37°C.
No or scant growth on glucose- or mannitolagar with nitrate as sole source of nitrogen.
Hydrolysis of starch: Negative.

Species No. III:

Morphology:
Vegetative rods: 0.6—0.7 μ × 1.5—3.5 μ ends rounded.
Spores: 0.7—0.9 μ × 1.0—1.2 μ ellipsoidal, paracentral to subterminal, thickwalled.
Sporangia: Some are definitely swollen, others are not. The rods in sporulated cultures swell and grow very thick but keep their original shape. Sporulating short rods may have a globular form at this stage. Thus, there is no local swelling at the site where the spore is placed, although some sporangia are spindle-shaped.

Variety a:
C 326, C 342
Grampositive.
No or only scant growth on nutrient agar at pH 7.3.
Maximum temperature for growth: 50°C.
No or only scant growth on glucose- or mannitolagar at pH 9.7 with nitrate as sole source of nitrogen.
Hydrolysis of starch: positive, wide zone of hydrolysis.

Variety b:
C 347, C 350.
Grampositive.
Moderate growth on nutrient agar at pH 7.3.
Maximum temperature for growth: 50°C.
Scant growth on glucose- or mannitolagar at pH 9.7 with nitrate as sole source of nitrogen.
Hydrolysis of starch: positive, wide zone of hydrolysis.

Variety c:
C 337, C 340.
Gramnegative or Gramvariable.
Moderate growth on nutrient agar at pH 7.3.
Maximum temperature for growth: 37 and 50°C, respectively.
Scant growth on glucose- or mannitolagar at pH 9.7 with nitrate as sole source of nitrogen.
Hydrolysis of starch: positive, wide zone of hydrolysis.

Variety d:
C 338, C 343, C 346, C 348, C 349.
Gramvariable or Gramnegative.
No or only scant growth on nutrient agar at pH 7.3.

Maximum temperature for growth: 45—50°C.:

No or only very scant growth on glucose- or mannitolagar at pH 9.7 with nitrate as sole source of nitrogen.

Hydrolysis of starch: positive, wide zone of hydrolysis.

Variety e:
C 324, C 355.
Grampositive.
No or only very scant growth on nutrient agar at pH 7.3.
Maximum temperature for growth: 45—50°C.
Moderate growth on glucose- or mannitolagar at pH 9.7 with nitrate as sole source of nitrogen.
Hydrolysis of starch: positive, wide zone of hydrolysis.

Variety f:
C 353
Gramnegative.
Moderate growth on nutrient agar at pH 7.3.
Maximum temperature for growth. 50°C.
Moderate growth on glucose- or mannitolagar at pH 9.7 with nitrate as sole source of nitrogen.
Hydrolysis of starch: positive, wide zone of hydrolysis.

Species No. IV:
(belonging to morphological group II according to Bergey).

Morphology:
Vegetative rods: 0.4—0.5 $\mu \times 2$—3 μ often in long chains.
Spores: 0.6—0.8$\mu \times 0.7$—0.9μ, oval, subterminal, Thickwalled, easly stained.
Sporangia: Definitely swollen, clavate.

Variety a:
C 303, C 354, C 357, C 366, C 367, C 371, C 375, C 378.
Gramnegative.
Moderate to good growth on nutrient agar at pH 7.3.
Maximum temperature for growth: 57°C.
Moderate to good growth on glucose- or mannitolagar at pH 9.7 with nitrate as sole source of nitrogen.
Hydrolysis of starch: positive, moderate zone of hydrolysis.

Variety b:
C 351, C 356, C 364, C 376, C 377, C 411
Gramvariable.
Otherwise like variety a.

Variety c:
C 358, C 410.
Grampositive.
Otherwise like variety a.

Species No. V:
(belonging to morphological group II according to Bergey)
C 365, C 412.

Morphology:
Vegetative rods: 0.3—0.4 $\mu \times 2$—4 μ, straight, some slightly bent
Spores: 0.6—0.7 $\mu \times 0.9$—1.2 μ, oval to ellipsoidal paracentral to terminal, thickwalled, easly stained, remnants of sporangia often adhering.
Sporangia: Definitely swollen, clavate to racket-shaped.
Grampositive.
Moderate to good growth on nutrient agar at pH 7.3.
Maximum temperature for growth: 57°C.
Good growth on glucose- or mannitolagar at pH 9.7 with nitrate as sole source of nitrogen.
Hydrolysis of starch: positive, moderate to wide zone of hydrolysis.

Species No. VI:
(belonging to morphological group II according to Bergey)

Morphology:
Vegetative rods: 0.25—0.35 $\mu \times 2.5$—5 μ. Slightly bent, ends pointed
Spores: 0.8—1$\mu \times 1.1$—1.3μ. Oval to ellipsodial, subterminal to terminal.
Sporangia: Definitely swollen, clavate to drumstick-shaped.

Variety a:
C 373
Gramnegative.
No or only scant growth on nutrient agar at pH 7.3.
Maximum temperature for growth: 50°C.
No or only very scant growth on glucose- or mannitolagar at pH 9.7 with nitrate as sole source of nitrogen.
Hydrolysis of starch: Positive, wide zone of hydrolysis.

Variety b:
C 325, C 413
Grampositive.
Moderate growth on nutrient agar at pH 7.3.

Maximum temperature for growth: 50°C.

No or only very scant growth on glucose- or mannitolagar at pH 9.7 with nitrate as sole source of nitrogen.

Hydrolysis of starch: Positive, wide zone of hydrolysis.

Species No. VII:

C 370.

This strain was tentatively classified as a separate species.

It is asporogenous or oligosporogenous and therefore cannot be placed in any morphological group.

It seems mostly related to the alkaline bacteria which belong to the morphological group II of the genus Bacillus (according to Bergey).

Vegetative rods: 0.3—0.5 $\mu \times 2$—5 μ, often in long chains, filamentous, ends slightly pointed and rounded.

Gramnegative.

Moderate to good growth on nutrient agar at pH 7.3.

Maximum temperature for growth: 57°C.

Good growth on glucose- or mannitolagar pH 9.7 with nitrate as sole source of nitrogen.

Hydrolysis of starch: Positive, moderate zone or hydrolysis.

The following characters are common to a number of strains belonging to the different varieties in the species I—IV:

Hydrolysis of gelatine: Positive

Hydrolysis of casein: Positive.

Glucose-nutrient agar slants.— Growth same as on nutrient agar, or heavier.

Soybean agar slants.— Growth more abundant and softer than on nutrient agar.

Tyrosine agar slants.— Growth same as on nutrient agar.

Nutrient broth.— Medium turbidity with abundant sediment.

No pellicle or pellicle thin and friable.

NaCl broth.— Good growth in 5 per cent concentration of NaCl; growth at 7 per cent.

Production of acetylmethylcarbinol.— Negative.

Reduction of nitrate to nitrite.— Positive.

Anaerobic growth in glucose.— Scant, if any, growth; pH 7.8 or higher at 14 days. (pH of the medium adjusted to 9 before inoculation).

On the basis of the inventors' taxonomic investigations the members of the genus Bacillus listed in Table I should be classified as it appears from Table II below.

TABLE II

Species:	Variety:	Strains:
I	a	C 300, C 301, C 360 C 372, C 374
	b	C 302, C 334
	c	C 323, C 339, C 352, C 369
	d	C 304, C 311, C 336
II		C 335, C 341
III	a	C 326, C 342
	b	C 347, C 350
	c	C 337, C 340
	d	C 338, C 343, C 346, C 348, C 349
	e	C 324, C 355
	f	C 353
IV	a	C 303, C 354, C 357, C 366, C 367, C 371, C 375, C 378
	b	C 351, C 356, C 364, C 376, C 377, C 411
	c	C 358, C 410
V		C 365, C 412
VI	a	C 373
	b	C 325, C 413
VII		C 370

All the strains referred to have also been grown on nutrient agar and soy bean agar and some of them on glucose nitrate agar and mannitol nitrate agar, for the purpose of observing the form, appearance and colour of the colonies, but as the relevance of these observations may be disputed, the results of the inventors' observations are not reported here.

However, the following Table III reviews the properties the variations of which form the basis for the division of varieties within the different morphological groups:

TABLE III

Species:	Var.:	Gram stain:	Growth on nutrient agar pH 7.3:	Maximum growth temp.:	Growth on nitrate (NO_3) as sole N-source	Hydrolysis of starch:	Width of zone of hydrolysis on starch agar	Maximum growth velocity at pH:
I	a	+	+++	50—55	scant	+	+	8.0—9.5
	b	+	++	40—50	scant	+	+++	8.0—8.5
	c	(+)	++	37—50	scant	+	+++	8.3—8.8
	d	+	++	37	scant	—	—	8.0—8.6
II		+	scant	37	scant	—	—	8.0—9.0
III	a	+	scant	50	scant	+	+++	8.3—9.0
	b	+	++	50	scant	+	+++	
	c	—, var.	++	37—50	scant	+	+++	
	d	—, var.	scant	45—50	scant	+	+++	
	e	+	scant	45—50	++	+	+++	8.5—9.0
	f	—	++	50	++	+	+++	
IV	a	—	+++	57	+++	+	++	8.0—9.0
	b	var.	+++	57	+++	+	++	8.0—9.0
	c	+	+++	57	+++	+	++	
V		+	+++	57	+++	+	+++	
VI	a	—	scant	50	scant	+	+++	8.5—9.2
	b	+	++	50	scant	+	+++	
VII		—	+++	57	+++	+	++	8.0—8.5

scant = poor

stain = colour

All the species and strains in Tables I and II have been cultivated with proteolytic enzyme production in view. This cultivation has been carried out both in shake flasks and in tanks in pilot plants with artificial aeration. The yields obtained have been determined by the well-known Anson hemoglobin method, cf Journal of General Physiology, 22, 79—89 (1939). One Anson unit means throughout this specification the amount of proteolytic enzyme digesting hemoglobin at a pH-value of 10.1 and a temperature of 25°C during a reaction time of 10 minutes with such an initial velocity that per minute there is formed such an amount of split products which cannot be precipitated with trichloroacetic acid that these split products give the same colour with phenol reagent as does one milliequivalent of tyrosine.

The general process for producing the proteolytic enzymes belongs to the known art in which the enzymes are produced by aerobic cultivation of bacteria in a nutrient medium containing assimilable carbon and nitrogen sources, but is characterised by maintaining a pH-value of the nutrient medium within the range of 7—12 during the cultivation, using species of the genus Bacillus able to grow and produce the proteolytic enzymes within the pH-range referred to, and recovering from the medium the proteolytic enzymes formed during the cultivation.

Experiments seem to show that it is convenient to carry out the cultivation at a pH-value of the culture medium within the range of 7.5 to 10.5.

The nutrient medium is made up in accordance with the principles of the known art. Suitable assimilable carbon sources are carbohydrates, such as sucrose, glucose, starch, flour from cereal grains, malt, rice, sorghum etc. The carbohydrate concentration may vary within rather wide limits, e.g. up to 25% and down to 1—5 %, but usually 8—10 % would be suitable, the percentage being calculated as dextrose. It has been found that the presence in the nutrient medium of carbohydrates will give rise to the formation of acidic components, resulting in a decrease of the pH-value during the cultivation. As it is essential to maintain a pH-value of the nutrient medium within the range of 7 to 12 during the cultivation, measurements should be taken that the pH-value does not fall below 7 for any essential period during the cultivation. In order to keep the pH-value within the required range, a limited amount of carbohydrates may be used together with a buffer substance which is able to maintain the required pH-value. It has been found that carbonates, and particularly sesquicarbonates, used in a concentration of up to 0.2 M in the medium, are able to create a pH-value of about 10.5 and 9.3, respectively.

Also other buffer systems, such as phosphate buffers, may be used.

It is also possible to initiate the cultivation with a low carbohydrate content and to add small amounts of carbohydrates successively during the cultivation.

A third possibility is to make use of automatic pH-control by addition of various basic-reacting substances used in this art.

The use of carbonates and sesquicarbonates as pH-controlling substances is very useful and it is surprising that it is possible during the cultivation to use these compounds in the concentrations referred to.

The nitrogen source in the nutrient medium may be inorganic and/or organic nature. Suitable inorganic nitrogen sources are nitrates and ammonium salts, and among the organic nitrogen sources there are quite a number known for use in fermentation processes and in the cultivation of bacteria. Illustrating examples are soy meal, cotton seed meal, peanut meal, casein, corn steep liquor, yeast extracts, urea and albumin.

Besides, the nutrient medium should naturally contain the usual trace substances.

The temperature at which the cultivation takes place is normally within the same range as in the known cultivation of known species of the genus Bacillus. Usually a temperature between 25 and 55°C is convenient. The temperature is preferably 30 to 40°C.

As the cultivation has to be carried out under aerobic conditions, it is, when using fermentation tanks, necessary to make use of artificial aeration. The amount of air is similar to that used in the known cultivation processes.

In general, maximum yields of the proteolytic enzymes will be obtained after a cultivation time of 1 to 5 days.

Although most of the experiments in connection with the production of proteolytic enzymes from the species and strains compiled in Table II, have been carried out in shake flasks or in tanks in pilot plants, use has also been made of surface growth. In such case the nutrient medium consisted of 10 g of wheat bran, 2 g Na_3PO_4, $12H_2O$ and about 10 ml water. Prior to inoculation the pH-value was adjusted to about 10 with 2 ml 1 N NaOH. On this medium strains C 309 and C 303 were cultivated by surface growth, and for both strains the yield of proteolytic enzymes was about 20 Anson units per kg of wheat bran at pH 10.

For cultivation of the species and strains compiled in Table II the following two media were used:

1) Medium BPFA with the following composition:

Potato flour	50	g per liter of tap water
Sucrose	50	g „ „ „ „ „
Barley flour	50	g „ „ „ „ „
Soy meal	20	g „ „ „ „ „
Sodium caseinate	10	g „ „ „ „ „
$Na_2HPO_4 12H_2O$	9	g „ „ „ „ „
Pluronic	0.1	g „ „ „ „ „

2) Medium BSX with the following composition:

Barley flour	100	g per litre of tap water
Soy meal	30	g „ „ „ „ „
Pluronic	0.1	g „ „ „ „ „

Both these media were adjusted to the desired pH-value by the addition of sesquicarbonate or sodium carbonate under sterile conditions. The word "Pluronic" is a Trade Mark for certain non-ionic surfactants.

The experiments in shake flasks were carried out in 500 ml shake flasks, each of the flasks containing 100 ml of the nutrient medium BPFA and BSX, respectively, which were sterilized beforehand by autoclaving for 90 minutes at 120°C and after the autoclaving the pH-value was adjusted to 9.3—10.5 with sodium sesquicarbonate. There were used four flasks for each bacterium, and samples from the culture media for determining the enzyme content expressed in Anson units were taken after cultivation in 3, 4, 5 and 6 days, respectively. The flasks were during the cultivation placed on a rotating table with 240 revolutions per minute.

In the following Table IV are compiled the maximum enzyme yields and the pH-value at that time.

TABLE IV

Species:	Variety:	Strains:	BPFA Anson units per kg.	pH of medium	BSX Anson units per kg.	pH of medium
I	a	C 300	19	6.5	19	9.5
		C 301	10	6.5	24	9.5
		C 360	20	6.3	30	9.5
		C 372	20	6.4	15	9.3
		C 374	11	6.3	18	9.3
	b	C 302	36	9.2	16	9.3
		C 334	38	8.6	10	9.5
	c	C 323	22	7.6	9	10.0
		C 339	38	9.3	19	9.2
		C 352	32	9.1	33	9.5
		C 369	115	9.3	16	9.3
	d	C 304	48	8.1	7	6.5
		C 311	67	9.4	11	9.7
		C 336	2	8.0	1	9.1
II		C 335	38	9.5	3	10.0
		C 341	42	9.2	3	9.9
IV	a	C 303	3	7.2	30	9.7
		C 354	17	9.0	11	9.1
		C 357	4	8.2	25	9.4
		C 366	5	8.7	30	9.5
		C 367	4	8.8	35	9.4
		C 371	2	8.5	40	9.4
		C 375	1	7.7	30	9.6
		C 378	2	8.5	25	9.3
	b	C 351	2	8.3	30	9.7
		C 356	3	7.7	20	9.7
		C 364	3	8.3	40	9.3
		C 376	10	9.3	20	9.7
		C 377	1	7.8	9	9.7
		C 411	0		15	
	c	C 358	2	7.1	25	9.6
		C 410	3		17	

TABLE IV (Continued)

Species:	Variety:	Strains:	BPFA Anson units per kg:	pH of medium:	BSX Anson units per kg:	pH of medium:
V		C 365 C 412	60 4	8.2 —	55 80	9.5 —
VII		C 370	115	9.0	45	9.3
III	a	C 326 C 342	47 67	9.5 8.9	5 10	9.8 9.4
	b	C 347 C 350	44 44	9.3 8.8	7 13	9.1 8.9
	c	C 337 C 340	78 22	8.0 9.4	13 7	9.6 9.8
	d	C 338 C 343 C 346 C 348 C 349	72 14 26 17 21	8.2 9.0 8.5 9.6 9.0	7 2 5 7 11	7.7 8.9 9.4 9.1 9.2
	e	C 324 C 355	97 60	8.9 9.1	8 6	9.5 9.2
	f	C 353	96	8.9	8	9.6
VI	a	C 373	12	5.3	1	6.4
	b	C 325 C 413	1 0	9.5 —	1 18	6.5 —

The two culture media BPFA and BSX have also been for cultivation in tanks under submerged conditions and artificial aeration, use being made of 550 liter stainless steel tanks. To illustrate such cultivations in pilot plant reference is made to the following Table V giving information of the strains used, the cultivation conditions and the results obtained.

TABLE V

Strain	C 324	C 335	C 339	C 347	C 351
Medium	BPFA	BPFA	BPFA	BPFA	BSX
pH-value before inoculation	9.3	10.2	10.5	10.2	10.2
Cultivation temperature in degrees Centigrade	34	34	34	34	34
Air, m^3 per minute	0.3	0.25	0.25	0.25	0.3
Cultivation time in hours	53	84	97	59	83
Final pH-value	8.3	9.2	9.1	8.9	9.35
Final proteolytic activity expressed in Anson units per kg of substrate	44	40	29	48	33

In other pilot plant cultivations use has been made of other strains with varying compositions of the cultivation medium. In the steel tanks referred to above strain C 303 has been cultivated in four runs under different conditions. The compositions of the cultivation media, the cultivation conditions and the results obtained are given in the following Table VI.

TABLE VI

Cultivation No.	1	2	3	4
Barley flour g/liter	100	100	150	200
Soymeal g/liter	30	30	45	60
Pluronic ml/liter	0.03	0.03	0.03	0.03
Na_2CO_3 (sterile addition before inoculation) to give	0.2 M	0.2 M	0.2 M	0.4 M
pH before inoculation	10.0	10.0	10.35	10.1
Cultivation temperature °C	34	34	34	34
Air m^3/minute	0.3	0.3	0.3	0.3
Cultivation time in hours	125	104	113	126
pH at maximum	9.3	9.3	9.6	9.3
Maximum Anson units per kg	67	80	77	66

The proteolytic enzymes can be recovered from the cultivation broth by subjecting the broth to centrifugation, precipitating the enzyme from the liquid thus obtained by addition of Na_2SO_4 or ethanol, separating the precipitate from the liquid by filtration with kieselguhr as filtration aid and drying the precipitate to form a powder containing the active proteolytic enzymes.

Numerous recovery processes are known in the art and the processes referred to are given by way of example only.

TABLE VII

Strain:	C 339	C 347	C 351
Starting material	250 kg culture liquid containing 25 Anson units per kg	250 kg culture liquid containing 45 Anson units per kg	600 ml culture liquid containing 32 Anson units per liter
Total amount of Anson units	6250	11250	19.2
Centrifugation	3000 rpm/30 min.	3000 rpm/30 min.	4000 rpm/30 min.
Precipitation	35°C 85 kg Na_2SO_4 standing one hour	35°C 85 kg Na_2SO_4 standing one hour	0°C 1200 ml C_2H_5OH
Filtration	2.25 kg Kieselguhr filter press	filter press	none (centrifugation 30 min. 4000 rpm washing with 600 ml C_2H_5OH at 0°C, centrifugation 4000 rpm/10 min.)
Drying	Drying chamber, 40°C	Drying chamber, 40°C	Vacuum, P_2O_5
Enzyme powder	4000 g containing 0.4 Anson units per g	1300 g containing 0.8 Anson units per g	9.7 g containing 1.3 Anson units per g
Yield	1600 Anson units = 25%	1020 Anson units = 9%	12.6 Anson units = 65%

The starting materials mentioned in Table VII had been produced by cultivation in 550 liter stainless steel tanks under artificial aeration. The nutrient media were adjusted to the initial pH-value by a 2 M Na_2CO_3 solution added under sterile conditions before the inoculation.

Testing of the proteolytic enzymes produced by the strains listed in Table II has shown that there are material differences between the enzymes regarding a number of their properties.

With regard to the proteolytic activity it seems that the enzymes may be divided into three groups or types when the proteolytic activity is measured at pH 12 and expressed in percentage of maximum activity, viz.

Type 1: 100 to 80 %
Type 2: 80 to 50 %
Type 3: 50 to 0 %

It is known in the art that calcium ions stabilize the activity of most of the proteolytic enzymes. The novel enzymes produced by the bacteria listed in Table I and divided into species and varieties in Table II have been tested with regard to the stabilizing effect of calcium ions in a concentration of 0.01 M at pH 10.5 or 11, and the stabilization has been incated in percentage of residual activity after standing 30 minutes at 50°C. The results of the enzyme type testing and the calcium ion stabilization effect are compiled in Table VIII, in which plus means that the residual proteolytic activity in the absence of calcium ions is below 80 % of the corresponding activity of the control in the presence of calcium ions, and minus means that the residual proteolytic activity in the absence of calcium ions is above 80 % of the corresponding activity of the control in the presence of calcium ions.

TABLE VIII

Species	Var.	Strains:	Enzyme-type	CA^{++} stabilisation
I	a	C 300, C 301, C 360, C 372, C 374	1	+++++
	b	C 302, C 334	1	++
	c	C 323, C 339, C 352, C 369	2	?+++
	d	C 304, C C 311, C 336	1	+++
II		C 335, C 341	2	++
III	a	C 326, C 342	3	?+
	b	C 347. C 350	3	+?
	c	C 337, C 340	3	++
	d	C 338, C 343, C 346, C 348, C 349	3	+????
	e	C 324, C 355	3	+?
	f	C 353	3	+
IV	a	C 303, C 354, C 357, C 366, C 367, C 371, C 375 C 378	1	------?
	b	C 351, C 356, C 364, C 376, C 377, C 411	1	-?---+
	c	C 358, C 410	1	-+
V		C 365, C 412	1	-?
VI	a	C 373	1	-
	b	C 325, C 413	1	??
VII		C 370	1	-

The proteolytic activity of the enzymes produced by the strains listed in Table VIII has been tested not only at pH 12, but also at lower pH-values to give a more detailed impression of the proteolytic activity at different pH-values and more information of the activity of the three types of enzymes. For the purpose of illustration reference is made, by way of example, to the accompanying drawings, in which:—

Figure 1 shows the proteolytic activity of the enzyme produced by the strain C 311, said enzyme belonging to type 1,

Figure 2 in the same manner shows the activity of the enzyme produced by strain C 335, said enzyme belonging to type 2, and

Figure 3 in the same manner shows the activity of the enzyme produced by the strain C 324, said enzyme belonging to type 3.

It should be understood that the purpose of the activity curves shown in the drawings is to illustrate in principle the difference in activity of the three types of proteolytic enzymes at varying pH-values and that the activity curve for each type may vary somewhat without losing its characteristic appearance.

The novel enzymes according to the invention have furthermore been subjected to the following tests:

a) *Stability against tripolyphosphate (TPP)*

The stability of the enzymes in a solution containing tripolyphosphate in an amount of 0.2 % was determined. The stability has been expressed as percentage of residual activity after 30 minutes at 50°C and at pH 10. The enzyme concentration was 0.1 Anson unit per liter and the method of analysis was the Anson method.

The results are compiled in the following Table IX.

The corresponding solutions with 0.01 M $CaCl_2$ showed in all cases a residual activity of 80 to 100 %.

b) *Stability against perborate.*

The activity of a solution containing the enzyme and sodium perborate in an amount of 0.1 % was determined. The stability was expressed as percentage of residual activity after 30 minutes at 50°C and pH 10. The enzyme concentration and the method of analysis were as in test a).

The results are compiled in the following Table IX.

c) *Stability against surfactants.*

The stability of solutions containing the enzyme and different surfactants was determined while using three typical surfactants in concentrations corresponding to those employed in washing solutions:

1. Soap	0.25	g per liter
2. DBS — an alkyl-aryl sulphonate (50%)	2.5	g per liter
3. TAS — tallow-alcohol-sulphate (25%)	5.0	g per liter.

The enzyme concentration was 0.1 Anson unit per liter. The test conditions were 30 minutes at 50°C at pH 10, and the method of analysis was the nitro-casein-method, cf E.v. Pechmann, Biochemische Zeitschrift, Bd. 321, 248—260 (1950).

The figures in Table IX are to be understood as follows:

The figure above the stroke shows the percentage of residual activity, when one performs the analysis immediately after the addition of the surfactant, i.e. this figure gives an indication of the initial rate of inactivation.

The figure under the stroke is the difference between this initial residual activity and the percentage of residual activity after 30 minutes.

d) *Temperature optimum.*

In Table IX is indicated the temperature in degrees Centigrade at which there was found maximum activity at pH 10. The method of analysis was the Anson method.

e) *Enzyme type.*

It has been found that all the enzyme preparations are inhibited instantaneously and completely by phenylmethylsulphonylfluoride, which means that all the enzymes have serine in the active centre.

f) *pH-Stability.*

In connection with five enzyme preparations the stability at different pH-values has been determined under the following conditions:

Standing: 24 hours at 25°C
pH-values: 5-7-8-10-12
Enzyme concentration: 0.2 Anson units per liter.

In Table IV is indicated the pH-range within which there was found a residual activity of 80 to 100 per cent.

Testing of the enzyme preparations produced by strains C 303 and C 347 against sodium sulphite has shown that the enzymes are not sensitive to this reducing agent, which might indicate that S—S—bridges are not essential for the tertiary structure of the enzymes.

TABLE IX

Strain	Enzyme preparation in powder form g	Activity Anson units per g at pH	Enzyme type	Stability					Temp. optimum °C	Serine	pH stability
				TPP	per-borate	surfactants					
						soap	DBS	TAS			
C 300	2	0.5 (10)	1	2	89	82/71	7/6	7/6	50	+	
C 301	2	0.7 (10)	1	4	47	50/47	35/31	4/2	50	+	
C 302	3	0.7 (10)	1	1	48	59/56	60/—	3/1	50	+	
C 303	600	3.0 (7.5)	1	91	95	85/50	40/30	23/18	60	+	6.0—10.5
C 304	2.5	0.3 (7.5)	1	2	4	57/56	34/31	3/0	50	+	
C 334	1500	0.5 (7.5)	1	95	39	100/83	90/27	93/73	40	+	
C 351	9	1.3 (7.5)	1	78	66	93/67	67/50	53/45	60	+	
C 354	42	0.6 (7.5)	1	94	82	77/33	60/38	60/53	60	+	
C 360	117	0.4 (7.5)	1	0	83	97/94	76/70	75/75	50	+	
C 364	456	0.9 (7.5)	1	90	80	81/31	27/11	16/8	60	+	
C 365	2000	0.4 (7.5)	1	94	86	100/0	72/30	92/48	60	+	
C 366	26	0.8 (7.5)	1	100	96	92/42	67/14	68/61	60	+	
C 367	500	2.2 (7.5)	1	94	100	88/2	58/34	71/52	+	60	5.0—11.0
C 370	3000	0.6 (7.5)	1	89	91	94/4	33/15	67/30	60	+	
C 371	50	1.0 (7.5)	1	95	93	97/60	70/44	93/57	60	+	
C 372	430	2.7 (7.5)	1	6	60	98/95	75/68	87/85	50	+	6.0—10.5
C 376	17	7.8 (7.5)	1	88	78	66/42	25/10	15/12	60	+	
C 377	213	1.9 (7.5)	1	93	86	100/10	59/27	74/59	60	+	
C 335	1500	0.3 (7.5)	2	60	29	95/51	40/32	78/71	55	+	
C 339	217	1.4 (7.5)	2	16	76	89/10	75/57	83/62	45	+	6.3—10.3
C 369	7.2	1.4 (7.5)	2	81	2	28/28	6/6	0/0	50	+	
C 347	573	2.0 (7.5)	3	0	36	81/78	94/71	67/63	40	+	6.3—11.0

From Table IX it will be seen that a number of the enzyme preparations show astonishing stability properties.

In general, the enzyme preparations or compositions according to the invention consist of a solid or liquid mixture of the proteolytic enzymes produced according to the invention and other components the amount and composition of which depend on the purpose and technical or scientific field within which the enzyme compositions are to be used. When the enzyme preparations or compositions according to the invention are in solid form they can consist of granules into which the enzymes are incorporated, for instance together with other enzymes or substances having other than enzymatic activity useful for the utility of the enzyme compositions. When the enzymes are not used in crystalline form, they may be accompanied by impurities of organic nature, such as proteins and carbohydrates from the culture medium.

The enzyme composition in liquid form can constitute solutions or suspensions which may contain stabilizers, if necessary.

Usually, the novel enzymes of the invention are used in small quantities. In view thereof the enzyme preparations or compositions for industrial use normally show an enzyme content not exceeding about 10 % by weight.

The novel enzymes according to the invention can for instance be used in washing compositions, dehairing compositions, in preparations for hydrolysis of proteins, in dish-washing compositions and as additives to septic tanks and installations for purifying sewage.

Quite a number of tests have been carried out in connection with the utility of the enzyme preparations according to the invention in washing compositions and washing processes.

The washing experiments were carried out while using the EMPA test strips or swatches 116 and 112, respectively. In other words, in the washing experiments there have been used test strips soiled with blood, milk, and carbon black (No. 116) or with cocoa, milk, and sugar (No. 112). The experiments were carried out with the two types of test strips separately and each experiment was repeated three times at 50°C and 60°C. The enzyme concentration in the washing solution was 0.048 Anson units per liter. EMPA test strips are manufactured and sold by Eidgenössische Materialprüfimg-sanstalt of Switzerland.

COMPOSITION A.

The detergent which should represent a heavy duty detergent had the following composition:

Nansa S (40% sodiumalkylarylsulfonate, 60% sodium sulphate)	250 g
Nonyl phenol, 10 EO	30 g
Soap (80%)	30 g
Sodiumtripolyphosphate	300 g
CMC (60%)	16 g
Sodiumcarbonate, anhydr.	80 g
Sodiumsulphate, anhydr.	74 g
Sodiumperborate ($NaBO_3$, 4 H_2O)	220 g
Total	1000 g

The word "Nansa" is a Trade Mark and "10 EO" means that the nonyl phenol contains 10 ethylene oxide groups per mole of nonyl phenol.

The other conditions were as follows:

Hardness of water in German units	10°
Fabric to water ratio	1:40
Time of experiment	30 minutes
pH-value	about 10
Detergent concentration:	4.0 g per liter of washing solution

The washing process carried out at 50°C was as follows:

By means of a pipette 20 ml of the enzyme solution with an activity of 0.288 Anson units per liter were added to a 150 ml beaker. Beforehand, the pH-value was adjusted to 10.0 and the temperature to 20°C. At zero time there were added 100 ml detergent solution adjusted to pH 10.3 and 56°C. The concentration of the detergent was 4.8 g per liter. The beaker was immediately placed in a water thermostat at 50°C and 6 circular EMPA-test swatches were added with a total weight of 3.0 g. Agitation was effected with a glass spatula during 10 seconds and the beaker was standing in the thermostat 30 minutes in total, agitation being effected during 10 seconds every four minutes. Then the wash solution was separated, and the pH-value was measured after cooling. The test swatches were rinsed in running tap water during 10 minutes and then dried between two towels and ironed. Every test swatch was sub-

jected to remission measurements in a Beckman spectrophotometer at 460 mμ. The word "Beckman" is a Trade Mark.

In addition to these experiments control experiments were carried out in which the enzyme solution was replaced by water.

In the experiments carried out at 60°C the temperature of the detergent solution was adjusted to 68°C.

The results of these experiments are compiled in the following table X

TABLE X

Enzyme from strain	Remission of EMPA test swatch					
	116			112		
	untreated	after soaking and rinsing		untreated	after soaking and rinsing	
		50°C	60°C		50°C	60°C
None	11.9	14.6	17.8	34.0	41.1	40.3
C 303		32.6	33.4		41.5	43.9
C 339		27.0	23.0		39.3	39.7
C 351		30.0	27.7		40.5	40.2
C 367		34.0	33.0		39.7	41.3
C 377		34.0	33.2		41.7	43.8
C 364		33.6	31.0		41.9	43.5
C 372		38.1	29.7		43.6	42.5
C 376		32.7	32.7		42.4	43.2
C 366		35.8	33.5		41.2	41.5

The remission values are average figures of measurements of each of the six test swatches in each of the experiments.

Another series of washing experiments has been carried out with EMPA-test swatches No. 116 previously treated with a solution containing an anionic surfactant and sodium perborate at a temperature of 40°C during 20 minutes. After the treatment the test swatches were rinsed and dried. The treatment with perborate results in a heavy fixation of the soiling, which would then be more difficult to remove.

COMPOSITION B

The detergent had the following composition:

Nonylphenol, 10 EO	80 g
Sodium tripolyphosphate	400 g
CMC (45%)	20 g
Sodium carbonate, anhydr.	150 g
Sodium sulphate, anhydr.	350 g
Total	1000 g

Further conditions:

Water	10° German hardness
Fabric to water ratio	1:40
Period	30 minutes
Temperature	45°C
pH	about 10
Detergent concentration	4.0 g per liter of wash solution

The procedure was as reported in the washing experiments mentioned above, except that the wash solution had a temperature of 50°C before it was mixed with the enzyme solution.

There were carried out three experiments with each of the enzymes, and the results appear from the following Table XI.

TABLE XI

Enzyme from strain	Remission of EMPA-test swatch No. 116	
	untreated	after soaking and rinsing
None	16.4	18.7
C 303		36.8
C 339		32.3
C 351		31.0
C 367		34.3
C 377		37.6
C 364		34.6
C 372		43.2
C 376		36.8
C 366		37.3

The remission values are average figures of the measurements of each of the six test swatches in each of the experiments, except that the values in connection with the experiments with enzymes from strain C 339 and C 351 are based on one measurement.

Experiments have also been carried out while using different enzyme concentrations in the wash solution, viz. in the interval between 0.02 and 0.16 Anson units per liter of wash solution. For the experiments EMPA test swatches No. 116 were used and the detergent was the same as that used in connection with the first mentioned washing experiments, viz.

Nansa S (40% sodiumalkylarylsulphonate, 60% sodiumsulphate	250 g
Nonylphenol, 10 EO	30 g
Soap (80%)	30 g
Sodium tripolyphosphate	300 g
CMC (60%)	16 g
Sodium carbonate, anhydr.	80 g
Sodium sulphate, anhydr.	74 g
Sodium perborate ($NaBO_3$, 4 H_2O)	220 g
Total	1000 g

The test conditions were as follows:

Water	10° German hardness
Fabric to water ratio	1:40
Period	30 minutes
Temperature	45°C and 60°C
pH	about 10
Detergent concentration	4.0 g per liter of wash solution

The procedure is the same as that reported in the first mentioned experiments, except that the concentration of the enzyme solution was varied so that the activity in the wash solution gets the following values:

0.02 — 0.04 — 0.08 — 0.16 Anson units per liter

Before the detergent solution was mixed with the enzyme solution it was adjusted to

50°C and 68°C, respectively, corresponding to a temperature of 45°C and 60°C, respectively, in the wash solution ready for use.

Each enzyme preparation was tested at both temperatures.

The results of the remission measurements are collected in the following Table XII.

TABLE XII

Enzyme from strain	Temperature °C	Remission of EMPA-test swatches 116				
		Anson units per liter of wash solution				
		0	0.02	0.04	0.08	0.16
C 303	45	15.0	25.8	28.8	33.5	36.8
C 339	45	16.02	25.8	29.7	32.5	36.0
C 351	45	15.7	26.0	30.8	34.2	37.3
C 367	45	14.0	25.2	28.3	31.7	36.3
C 377	45	16.5	29.2	32.2	36.8	41.7
C 364	45	15.3	27.2	29.3	33.7	36.7
C 372	45	16.2	33.0	36.6	42.0	46.1
C 376	45	17.5	30.0	33.7	36.3	39.8
C 366	45	16.7	31.8	34.0	38.3	46.7
C 303	60	19.2	29.8	32.0	34.1	36.1
C 339	60	16.9	20.9	21.7	25.4	28.8
C 351	60	17.4	27.0	28.8	32.4	34.4
C 367	60	17.3	28.8	30.3	32.6	34.1
C 377	60	17.4	27.0	28.8	32.4	34.4
C 364	60	20.8	28.7	29.8	33.0	33.5
C 372	60	17.0	21.3	23.7	25.7	29.1
C 376	60	19.0	28.4	31.4	33.6	35.5
C 366	60	19.0	32.0	32.5	35.0	38.9

The remission values are average figures from the six measurements.

Finally, there have been carried out the following

Storage stability experiments (shelf life)

Each enzyme preparation was mixed in a Turbola mixer with the detergent composition A. Corresponding experiments were carried out with the same detergent, except that the perborate was substituted by anhydrous sodium sulphate. The water content in the detergent not containing perborate was 2.7%. The water content in the detergent containing perborate was naturally correspondingly higher because the perborate contains about 47 % water of crystallization.

All the mixtures were analyzed for proteolytic activity immediately after they were made and then placed in sealed glass containers at 40°C. The analysis for proteolytic activity was repeated with certain intervals. The analyses were carried out as follows:

From twelve different places in the glass containers there was taken a sample weighing 12.5 g which was transferred to a one liter volumetric flask. In respect of the perborate-containing mixture there were added 4.4 g sodium sulphite for the purpose of neutralizing the perborate. Deionized water was added up to the mark, and the solution was then kept for 30 minutes at 25°C while stirring mechanically. Then the proteolytic activity was determined in Anson units.

The mixtures tested and the residual proteolytic activity measured at the various periods of storage have been compiled in the following Table XIII.

TABLE XIII

Detergent plus enzyme from strain	Perborate	Storing periods. Anson units per gram of enzyme and percentage of activity after storing at 40°C in: 0 days AU/g	0 days Act. %	1 day AU/g	1 day Act. %	3 days AU/g	3 days Act. %	5 days AU/g	5 days Act. %	8 days AU/g	8 days Act. %	14 days AU/g	14 days Act. %	21 days AU/g	21 days Act. %	28 days AU/g	28 days Act. %
C 303	–	2.90	100	2.94	101	2.85	100			3.00	103	2.95	101	2.80	97	3.00	103
	+	2.90	100	2.78	96	2.90	100			2.50	86	2.50	86	1.86	67		
C 367	–	4.20	100	4.00	95	3.60	86			3.72	88	3.80	90	3.95	94		
	+	4.20	100	4.20	100	4.30	80			2.30	55	2.00	47	1.90	45		
C 377	–	2.65	100	2.50	94	2.38	90			2.40	90	2.50	94	2.41	90		
	+	2.65	100	2.65	100	2.30	86			1.62	61	0.37	52	1.44	54		
C 364	–	0.70	100	0.70	100			0.72	103	0.72	103	0.66	94	0.70	100		
	+	0.70	100	0.70	100			0.72	103	0.57	81	0.46	66	0.43	61		
C 372	–	2.97	100	2.97	100			3.00	100	3.00	100	2.94	100	3.00	100		
	+	2.84	100	2.57	90			2.10	74	2.10	74	1.40	49	1.03	36		
C 376	–	8.60	100	7.70	90			8.60	100	8.60	100	8.00	93	8.10	94		
	+	(7.90)		8.20	96			7.70	90	7.70	90	7.00	81	5.50	64		
C 366	–	0.90	100	0.86	95			0.94	104	0.94	104	0.87	97	0.85	95		
	+	0.90	100	0.70	78			0.70	78	0.70	78	0.71	79	0.68	76		

AU/g = Anson units per gram

Act. % = percentage of residual proteolytic activity

The stability of some of the enzymes in the presence of perborate is considerable.

Many of the enzymes according to the invention are useful not only in the detergent compositions referred to in the above experiments, but actually useful in all kinds of detergent compositions. Such compositions may contain water-soluble soaps, anionic synthetic detergents, such as water-soluble salts of organic sulphuric reaction products, non-ionic synthetic detergents, such as compounds produced by condensation of alkylene oxide groups with an organic hydrophobic compound, ampholytic synthetic detergents and other synthetic detergents. Also builders may be present in the detergents and the enzymes according to the experiments may be combined with such builders. Examples of builders are carbonates, borates, phosphates, polyphosphates, silicates and sulphates of the alkali metals, preferably sodium.

Also organic alkaline builders may be present and combined with the enzymes according to the invention, if convenient.

The enzyme preparations or compositions according to the invention for use in the detergent industry may also contain other enzymes showing utility in the washing processes. Quite a number of such enzymes are known in the art.

When the enzyme preparations or compositions according to the invention are intended for use as an active constituent of detergent compositions, the enzyme preparations are usually marketed as a powder, of which the active enzyme or enzymes constitute a minor amount, the balance of the powder consisting of inorganic salts, such as sodium sulphate, calcium phosphate and sodium chloride, sometimes together with other substances forming constituents of the final detergents.

As proteolytic enzymes have already found use as constituents of detergent compositions, all persons skilled in the art will know how to make the enzyme preparations or compositions according to the invention suited for use in detergent compositions.

It is an essential advance for use in detergent compositions that many of the enzymes according to the invention show optimal proteolytic activity at elevated pH-values and improved stability in the presence of perborates.

The proteolytic enzymes produced according to the invention may also form an active ingredient in dish-washing agents. In dish-washing, proteolytic activity at a relatively high pH value is desirable.

As it has been mentioned in the foregoing the enzyme preparations or compositions according to the invention can also be used for the purpose of dehairing skins and hides. In the old dehairing process the hides were placed in a bath containing calcium hydroxide and sodium sulphide and having a pH-value of about 12. This dehairing process is detrimental to the hairs which might be of commercial value.

During recent years use has been made of an enzymatic dehairing process, in which use has been made of proteolytic enzymes, and the dehairing has been carried out at a lower pH-value of 7 to 10, which does not affect the quality of the hairs. On the other hand, no material swelling of the skins or hides is obtained as it was in the old dehairing process, which makes difficulties in the further processing of the skins or hides.

These difficulties are known in the art, and it has been proposed to use proteolytic enzymes showing sufficient activity at a higher pH-value than 10.

As some of the enzymes according to the invention show optimal proteolytic activity at a pH-value up to 12, these enzymes are well suited for use in dehairing processes. The following experiments are intended to illustrate the utility of some of the enzymes according to the invention for dehairing.

A salted cowhide (the butt) is sliced in pieces measuring about 20 × 4 cm. The pieces are steeped 24 hours and fat and meat are scraped off. The pieces of hide are then placed in 400 ml of different enzyme solutions contained in glasses having a volume of 500 ml. The glasses are incubated at 30°C for 24 hours. The pieces are then removed from the solutions and the hairs are scraped off with a piece of Plexiglass. The word "Plexiglass" is a Trade Mark. The dehairing effect is evaluated in accordance with the following scale:

1. Easy and complete removal of the hairs.
2. Easy removal of the hairs, but spots of hairs remained on the hide.
3. No or difficult removal of the hairs.

The proteolytic enzyme solutions used contain 1 g calcium hydroxide per 130 g of water. The amount of enzymes appears from the below Table XIV, also indicating the pH-values at the beginning and the end of the dehairing process together with the results thereof.

TABLE XIV

No.	1	2		3		4	
Enzyme from strain	control	C 303		C 367		C 372	
Amount of enzyme Anson units	0	0.5	5	0.5	5	0.5	5
Initial pH-value	11.9	11.9	11.9	11.9	11.8	12.0	11.9
Final pH-value	11.9	11.8	11.8	11.8	11.8	11.9	11.8
Dehairing result	3	1	1	2	2	3	1

WHAT WE CLAIM IS:—

1. An enzyme preparation containing at least one proteolytic enzyme of the serine type produced by cultivation of species of the genus Bacillus, the said enzyme showing optimal proteolytic activity against hemoglobin in the presence of urea at a pH-value above 9.

2. An anzyme preparation according to claim 1, wherein the proteolytic activity of the enzyme when measured at pH 12 by the Anson-method is in the range of from 80 to 100% of maximum activity.

3. An enzyme preparation according to claim 1, wherein the proteolytic activity of the enzyme when measured at pH 12 by the Anson-method is in the range of from 50 to 80% of maximum activity.

4. An enzyme preparation according to claim 1, wherein the proteolytic activity of the enzyme when measured at pH 12 by the Anson-method is in the range of from 0 to 50% of maximum activity.

5. A process for producing the proteolytic enzyme(s) contained in the preparation of any one of claims 1 to 4 by aerobic cultivation of bacteria in a nutrient medium containing assimilable carbon and nitrogen sources, which comprises the cultivation of protease-producing species of the genus Bacillus isolated from nature on nutrient media with a pH-value within the range of 9 to 11, the maintenance of the pH-value of the nutrient medium within the range of from 7 to 12 during the main period of the cultivation, and the recovery of the proteolytic enzyme(s) formed during the cultivation.

6. A process according to claim 5, wherein, during the main period of the cultivation, the pH-value of the nutrient medium is maintained within the range of from 7.5 to 10.5.

7. A process according to claim 5 or 6, for producing the proteolytic enzymes contained in the preparation of claim 1, which comprises the cultivation of one or more of the species of the genus Bacillus deposited at the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen (Scotland) under Nos. 10144 to 10148 and 10281 to 10327.

8. A process according to claim 5, 6 or 7 for producing the proteolytic enzymes contained in the preparation of claim 2, which comprises the cultivation of one or more of the bacteria deposited at the Research Station referred to in claim 7 under Nos. 10144 to 10148, 10281, 10284, 10286, 10288, 10301, 10304, 10306 to 10313 and 10315 to 10327.

9. A process according to claim 5, 6 or 7, for producing the enzymes contained in the preparation of claim 3, which comprises the cultivation of one or more of the bacteria deposited at the Research Station referred to in claim 7 under Nos. 10282, 10287, 10291, 10293, 10302 and 10314.

10. A process according to claim 5, 6 or 7 for producing the proteolytic enzymes contained in the preparation of claim 4, which comprises the cultivation of one or more of the bacteria deposited at the Research Station referred to in claim 7 under Nos. 10283, 10285, 10289, 10290, 10292, 10294 to 10300, 10303 and 10305.

11. An enzyme whenever produced by the process of any one of claims 5 to 10.

12. The use of the enzyme preparation in any one of claims 1 to 4 in a detergent or washing composition.

13. The use of the enzyme preparation in any one of claims 1 to 4 in dehairing compositions.

14. Enzyme preparations comprising new enzymes showing a pronounced and useful proteolytic activity at high alkalinities, substantially as hereinbefore described.

15. A process for producing proteolytic enzyme preparations substantially as hereinbefore described.

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Fig. 1

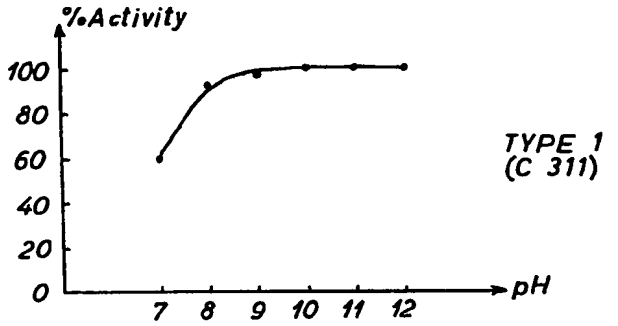

Fig. 2

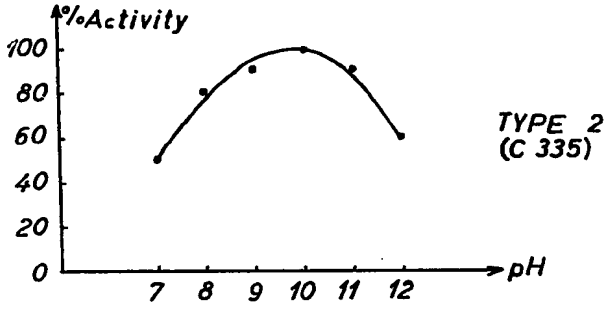

Fig. 3